PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		**Docket No.:**	**4247-101**
Applicant:	**XIAO, Xiao**	**Examiner:**	**Whiteman, B.A.**
Application No.:	**09/845,416**	**Art Unit:**	**1635**
Date Filed:	**April 30, 2001**	**Confirmation No.:**	**4144**
Title:	**DNA SEQUENCES COMPRISING DYSTROPHIN MINIGENES AND METHODS OF USE THEREOF (as amended)**	**Customer No.:**	**23448**

FACSIMILE TRANSMISSION CERTIFICATE
ATTN: Examiner Brian A. Whiteman
Fax No. (703) 872-9306

I hereby certify that this document is being filed in the United States Patent and Trademark Office, via facsimile transmission to Commissioner for Patents, Mail Stop Amendment, PO Box 1450, Alexandria, VA 22313-1450, on August 25, 2004 to United States Patent and Trademark Office facsimile transmission number (703) 872-9306.

44
Number of Pages

Steven J. Hultquist

August 25, 2004
Date of Transmission

DECLARATION OF XIAO XIAO UNDER 37 CFR §1.131 IN UNITED STATES PATENT APPLICATION NO. 09/845,416

I, XIAO XIAO, being duly sworn, depose and say:

1. THAT I am the sole inventor of the subject matter disclosed and claimed in U.S. Patent Application No. 09/845,416 filed in my name on April 30, 2001 for "DNA SEQUENCES ENCODING DYSTROPHIN MINIGENES AND METHODS OF USE THEREOF" ("Present Application").

2. THAT the Present Application variously claims an isolated nucleotide sequence comprising a dystrophin minigene encoding a protein having the following features: (a) a N-terminal domain; (b) four

to six rod repeats; (c) an H1 domain of a dystrophin protein and an H4 domain of the dystrophin protein; and (d) a cysteine-rich domain ("Invention").

3. THAT the Present Application claims the priority of U.S. Provisional Patent Application No. 60/200,777 filed in my name on April 28, 2000 for "DNA SEQUENCES ENCODING DYSTROPHIN MINIGENES AND USE THEREOF" ("Xiao Provisional Application") and that I am the sole inventor of the subject matter disclosed in the Xiao Provisional Application.

4. THAT I am aware that claims to the Invention in the Present Application have been rejected by the Patent and Trademark Office in a May 25, 2004 Office Action under 35 USC §102(e) as anticipated by Chamberlain et al. U.S. Patent Application No. 10/149,736 filed November 25, 2002 and published on November 20, 2003 as U.S. Patent Publication No. 2003/0216332, claiming priority of U.S. Provisional Patent Application No. 60/238,848 filed October 6, 2000 ("Chamberlain").

5. THAT the Invention was made prior to the October 6, 2000 effective date of Chamberlain as evidenced by the subject matter that I have described in the Xiao Provisional Application, including the following disclosures therein:

(a) page 4, lines 17-21 ("[a]lthough the [prior art] mini-genes contained ... 1 to 3 central rod repeats, they were ... insufficient to protect muscle from dystrophic pathology") excluding 1 to 3 rod constructs from the scope of the invention;

(b) page 6, lines 2-4 ("[t]he present invention provides the dystrophin gene which can be successfully reduced to approximately one third (1/3) of its 11 kb full-length coding sequence, without compromising essential functions in protecting muscles from dystrophic phenotypes");

(c) page 7, lines 3-6 ("Dystrophin has four major domains: the N-terminal domain (N), the cysteine-rich domain (CR), the C-terminal domain (CT) and the central rod domain, which contains 24 rod repeats (R) and 4 hinges (H). The mini-dystrophin genes were constructed by deleting a large portion of the central rods and nearly the entire CT domain (except the last 5 amino acids");

(d) page 53, lines 4-6 ("novel truncated dystrophin genes, which are small enough to be packaged into AAV vectors, and yet retain the essential functions needed to protect muscle from the pathological symptoms");

(e) page 55, lines 8-10 ("novel dystrophin constructs created by extensive deletions in the central rod domain, plus extensive deletions in the C-terminal domain of the human dystrophin cDNA");

(f) page 55, lines 12-13 ("[t]he mini-dystrophin genes are smaller than the 5-kilobase packaging limit of AAV viral vectors");

(g) page 56, lines 8-9 ("a major portion of the rod domain is dispensable") and lines 14-15 ("[w]e have created by rational design several mini-genes, in each deleting up to ¾ of the central rod domain"), which within the limits of accuracy of "rational design" can involve deletion of up to 20 of the 24 naturally-occuring rod repeats (in which ¾, when expressed as a decimal fraction of relevant significant digits (= 0.8), yields a rod domain deletion portion = 0.8 x 24 rods = 19.2 rods, which in turn due to the whole number character of repeat units requires deletion of 20 of the 24 naturally-occuring rods, to produce a 4-rod construct);

(h) page 60, lines 5-7 ("[t]o accommodate as many rod units in the central domain without exceeding the AAV vector packaging limit, we have for the first time deleted the entire C-terminus (819 bp) without sacrificing the primary functions of dystrophin") and lines 19-22 ("Mini-genes Δ3849" and "Mini-gene Δ3990" as 5-rod constructs, and "Mini-gene 4173" as a 6-rod construct);

(i) page 62, lines 7-8 ("[t]he entire gene expression cassettes can be readily packaged into adeno-associated virus (AAV) vectors"); and

(j) page 62, lines 12-14 ("the present invention further defines the minimal functional domains of dystrophin and provides ways to optimize and create new versions of mini-dystrophin genes").

6. THAT disclosure cited by the Examiner in the May 25, 2004 Office Action, at page 56 of the Xiao Provisional Application ("[t]o ensure sufficient physical flexibility of the protein, all of our mini-dystrophins still retain at least 5 rod repeats...in the central rod domain") describes specific illustrative constructs selected for protein physical flexibility, within the scope of broader disclosure of the Invention evidenced by the textual portions of the Xiao Provisional Application quoted in sub-paragraphs (a)-(j) of the preceding Paragraph 5 of this Declaration.

7. THAT disclosure cited by the Examiner in the May 25, 2004 Office Action, at page 60 of the Xiao Provisional Application ("the mini-dystrophin genes reported here accommodated at least 5 rod repeats") describes specific illustrative constructs selected for protein physical flexibility, within the scope of broader disclosure of the Invention evidenced by the textual portions of the Xiao Provisional Application quoted in sub-paragraphs (a)-(j) of the preceding Paragraph 5 of this Declaration.

8. THAT the April 28, 2000 filing date of the Xiao Provisional Application (containing broad disclosure of the Invention, including the textual portions of the Xiao Provisional Application quoted in sub-

paragraphs (a)-(j) of the preceding Paragraph 5 of this Declaration) is prior to the October 6, 2000 effective date of Chamberlain.

9. THAT the broad disclosure of the Invention in the Xiao Provisional Application evidences the constructive reduction to practice of the Invention as of the April 28, 2000 filing date of the Xiao Provisional Application, involving a mini-dystrophin gene encoding a protein having a N-terminal domain, a cysteine-rich domain, hinge domains H1 and H4, and a number of rod repeats defined by deletion of a "large portion" (page 7, lines 3-6)/"major portion" (page 56, lines 8-9) of the 24 naturally-occuring rod repeats, producing a truncated form of the dystrophin gene with "extensive deletions in the central rod domain" (page 55, lines 8-10) that (A) excludes 1 to 3 rod constructs (page 5, lines 17-21), (B) includes deletion of "up to ¾ of the central rod domain" (page 56, lines 14-15), which within the limits of accuracy of "rational design" (page 56, line 15) can involve deletion of up to 20 of the 24 naturally-occuring rod repeats, yielding a 4-rod construct, (C) includes 5-rod and 6-rod constructs (page 60, lines 19-22), and (D) is sufficiently small for packaging in an AAV vector without loss of function (page 53, lines 4-6)

10. THAT the broad disclosure of the Invention in the Xiao Provisional Application identified in the preceding Paragraph 9 of this Declaration also evidences my conception of the Invention as of the April 28, 2000 filing date of the Xiao Provisional Application, prior to the effective filing date of Chamberlain.

11. THAT as additional evidence of conception of the Invention prior to the effective date of Chamberlain, attached in **Exhibit 1** hereof are true and exact copies of pages 1 (Face Page), 2, 81-82, 104 and 105 of a grant application submitted to the U.S. Department of Health and Human Services, in which relevant dates are blacked out, but which dates are prior to the effective date of Chamberlain.

12. THAT the pages of the grant application referred to in the preceding Paragraph 11 of this Declaration, and attached in Exhibit 1 of this Declaration, contain the following disclosure: (i) an identification of a proposed grant project entitled "Improving Muscle Function Through Gene Delivery" (page 1 (Face Page)); (ii) an identification of me ("Dr. Xiao") as responsible for Project 1, entitled "Adenoassociated virus (AAV) vectors to improve mature muscle function by gene delivery" (page 2); (iii) disclosure of a dystrophin mini-gene lacking the central rod domain, with only one central rod (page 81-82); (iv) hypothesis of a single-rod dystrophin mini-gene as improving the function of dystrophin-deficient muscle when delivered by AAV vectors (page 104) and (v) description of alternative work in the event that the single rod construct were not to produce significant functional recovery, including removal of a portion of

the C-terminal region to "empty some space for additional rod domains to be incorporated" (page 105), whereby numbers of rods greater than one would be evaluated in constructs compatible with AAV vectors.

13. THAT following my original conception of the Invention, I undertook various activities and efforts to advance the Invention, including: (i) my submission in February, 2000 to the American Society of Gene Therapy (ASGT) of an Abstract ("Efficient Functional Correction of Muscular Dystrophy in mdx Mice by AAV Vectors Carrying Novel Human Mini-dystrophin Genes") describing "for the first time the successful gene therapy of DMD using AAV vectors" using "a series of novel mini-dystrophin genes (3.8 kb to 4.2 kb) that readily package into AAV along with an MCK (muscle-specific creatine kinase) promoter to assure tissue-specific transgene expression"), which was published on-line by the ASGT on May 1, 2000 in Molecular Therapy, Vol. 1, No. 5, May 2000, Part 2, a true and exact copy of which is attached in **Exhibit 2** hereof, (ii) my corresponding oral presentation on the Invention and appertaining research results at the ASGT Third Annual Meeting, at 2:00 PM on June 1, 2000, (iii) my submission in July, 2000 to the Proceedings of the National Academy of Sciences of the United States of America of a manuscript entitled "Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in *mdx* mouse model," which was published in such Proceedings in its December 5, 2000 issue, Vol. 97, No. 25, pages 13714-13719, a true and exact copy of which is attached in **Exhibit 3** hereof; and (iv) my filing on April 30, 2001 of the Present Application.

14. THAT this Declaration is submitted by me in order to present evidence of my conception and making of the Invention prior to the effective date of Chamberlain, and evidence of my activities and efforts in advancement of the Invention, in order to remove Chamberlain as prior art under 35 USC §102(e) against claims of the Present Application that have been rejected by the U.S. Patent and Trademark Office in the May 25, 2004 Office Action on the basis of Chamberlain.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the Present Application or any patent(s) issued thereon.

PATENT APPLICATION

Date: 8-24-2004

XIAO XIAO

Form Approved Through [redacted]
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK–FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
Improving Muscle Function through Gene Delivery

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES *(If "Yes," state number and title)*
PA-[redacted]-044 Pathogenesis and Therapy of the Muscular Dystrophies

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*	3b. DEGREE(S)	3c. SOCIAL SECURITY NO.
Hoffman, Eric P.	Ph.D.	

3d. POSITION TITLE
Associate Professor

3e. MAILING ADDRESS *(Street, city, state, zip code)*
University of Pittsburgh
Molecular Genetics and Biochemistry
W1240 Biomedical Science Tower
Pittsburgh, PA 15261

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Molecular Genetics and Biochemistry

3g. MAJOR SUBDIVISION
Medicine

3h. TELEPHONE AND FAX *(Area Code, number and extension)*
TEL: (412) 648-9024
FAX: (412) 624-1401

E-MAIL ADDRESS:
eric@hoffman.mgen.pitt.edu

4. HUMAN SUBJECTS	4a. If "Yes," Exemption no. or IRB approval date	4b. Assurance of compliance no.	5. VERTEBRATE ANIMALS	5a. If "Yes," IACUC approval date	5b. Animal welfare assurance no.
☒ No ☐ Yes	☐ Full IRB or ☐ Expedited Review		☐ No ☒ Yes	pending	A3187-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year–MM/DD/YY)*		7. COST REQUESTED FOR INITIAL BUDGET PERIOD		8. COST REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs
[redacted]	[redacted]	872,799	1,126,941	4,618,180	5,886,497

9. APPLICANT ORGANIZATION
Name: University of Pittsburgh
Address: Office of Research
350 Thackeray Hall
Pittsburgh, PA 15260

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
Forprofit → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE: 01

12. ENTITY IDENTIFICATION NUMBER: 25-0965591

Congressional District: 14th

13. ADMINISTRATIVE OF[illegible]
Name: Michael M
Title: Director
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* 2 page Biosketch
→ need to reduce PIs
After Other Support
PD first, then others

OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
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15. PRINCIPAL INVESTIG
I certify that the statements
knowledge. I am aware that
subject me to criminal, civil,
for the scientific conduct of t
grant is awarded as a result

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
[signature]
DATE: [redacted]

16. APPLICANT ORGANIZ[illegible] AND ACCEPTANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*
[signature]
DATE: [redacted]

BB Principal Investigator/Program Director (Last, first, middle): Hoffman, Eric P.

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Muscle is the largest organ system of the body, comprising approximately 30% of body mass. Muscle is considered one of the most adaptable tissues, responding quickly to use and disuse, and is capable of regeneration, hypertrophy, and alteration of metabolic status over relatively short periods of time.

Muscle dysfunction is an important human health problem. Inherited disorders of muscle, composed primarily of the muscular dystrophies, are some of the most common and most devastating inborn errors of human metabolism. Weakness due to aging (sarcopenia), and during space travel are also major concerns regarding the loss of muscle function.

There are clearly many advantages of muscle as an organ system in which to test the ability of exogenously added genes to modulate cellular function. However, as the muscle gene delivery field has expanded and matured over the last few years, significant hurdles facing practical applications of this technology have become recognized. It is these remaining hurdles that are the focus of this program project.

In this program project, we combine the complementary expertise of a group of seven independent investigators who have a track-record of synergistic collaboration, and commitment to the field of muscle, muscle disease, and muscle gene delivery. The interdependency of the different projects and sponsoring laboratories is evident through the extensive collaborative preliminary data that is presented, a long history of co-authored publications, and established shared training grants, group meetings, and journal clubs.

Research projects are as follows: Project 1 (Dr. Xiao), Adenoassociated virus (AAV) vectors to improve mature muscle function by gene delivery. Project 2 (Dr. Clemens), Long-term rescue of muscle function by dystrophin delivery using novel adenoviral vectors. Project 3 (Dr. Huard), Definition and circumvention of maturation-dependent infectivity of muscle by adenovirus. Project 4 (Dr. Hoffman), Development of targeting ligands for systemic delivery to muscle. Three Cores support the research projects; Administrative Core (Dr. Hoffman), Muscle Physiology Core (Dr. Watchko), and Mouse Breeding Core (Dr. Clemens).

PERFORMANCE SITE(S) (organization, city, state)

University of Pittsburgh, Pittsburgh, PA 15261

Children's Hospital of Pittsburgh, Pittsburgh, PA 15213

Magee Womens Hospital, Pittsburgh, PA 15213

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Eric P. Hoffman, Ph.D.	University of Pittsburgh	PI/PD
Paula Clemens, Ph.D.	University of Pittsburgh	PI
Leaf Huang, Ph.D.	University of Pittsburgh	Co-I
Johnny Huard, Ph.D.	Children's Hospital of Pittsburgh	PI
Marcia Ontell, Ph.D.	University of Pittsburgh	Co-I
Jon Watchko, M.D.	Magee Womens Hospital	PI
Xiao Xiao, ph.D.	University of Pittsburgh	PI

RESEARCH PLAN

A. Specific Aims:

Recombinant adeno-associate virus (rAAV) vectors are based on defective human parvoviruses. rAAV vectors have attracted attention due to their nonpathogenicity, possible genomic integration, transduction of quiescent cells, and apparent lack of cellular immune reactions. In contrast to other viral vectors, rAAV is capable of efficiently bypassing the myofiber basal lamina and transducing mature muscle cells (see Project 3; Dr. Huard). We and others have successfully demonstrated that rAAV vectors harboring a foreign gene, such as LacZ, can achieve highly efficient and sustained gene transfer in mature muscle of immunocompetent animals for more than 1.5 years without detectable toxicity. Vector integration into the host DNA and lack of CTL reaction against transduced cells support long term transgene expression.

Recently, we have significantly improved vector production methods to generate high titer and high quality rAAV vectors. However, despite success of rAAV for gene delivery in muscle using numerous reporter genes as well as therapeutic genes for metabolic disease and secreted proteins, no attempt to use this vector system to rescue muscle function loss caused by genetic deficiencies has been reported.

A major disadvantage of rAAV vectors is their 5-kb packaging size limit for foreign DNA. This has excluded its use for the major muscle disease gene, dystrophin (> 10 kb) in Duchenne muscular dystrophy (DMD). Indeed, even most truncated "Becker-like" forms of the dystrophin gene have been too large for rAAV vectors to accommodate.

In this project, we will use two animal model systems to test the hypothesis that functional rescue of muscle can be accomplished by genetic complementation of inherited muscular dystrophies. The first, the hamster model of δ-sarcoglycan (δ -SG) deficiency[85, 97], will be used to test our ability to deliver the normal δ-SG gene and restore muscle function. Complementation of the biochemical defect will be studied at the genetic, biochemical, histological, and functional levels. The second set of specific aims use the mouse model of dystrophin-deficiency[55] to test the efficacy of a highly truncated dystrophin gene.

Aim 1. Hypothesis: muscle deficient in δ-sarcoglycan (δ-SG) can be functionally rescued by genetic complementation using AAV vectors.

a. ***Intra-muscular injection of rAAV containing the δ-sarcoglycan gene driven by a CMV promoter will show functional rescue of muscle tissue.***

Dr. Hoffman's laboratory (Project 4) has been instrumental in defining human disorders resulting from SG defects, and has cloned the human δ-SG gene into AAV for use in this project. We will produce high titer rAAV-δ-SG virus, and test for efficiency of gene delivery, persistence of DNA in myofibers, and expression levels as a function of time from injection. Muscle functional improvement will be studied through histological studies (in collaboration with Dr. Hoffman's laboratory), and physiological studies (Muscle Physiology Core).

Any inflammation associated with δ-SG expression will be studied with the assistance from Dr. Clemens (Project 2). If inflammation appears to limit the persistence of δ-SG expression, and improvement of muscle function, then immune suppressants (FK506, cyclosporin) will be administered, and muscle function tested as above.

b. ***Persistence of gene expression in the hamster model will be improved with muscle-specific promoters.***

We anticipate that the CMV promoter used above will not prove ideal for muscle gene delivery. First, the promoter has been shown to be inactivated in other tissues. Second, the expression of δ-SG in non-muscle cells (APCs) may elicit an immune response against the vector transduced cells. For this reason, we will use muscle specific promoters (such as muscle-specific creatine kinase (MCK) promoter) to drive the SG gene in rAAV vectors. Dr. Clemens (Project 2) has substantial experience in using MCK promoter in adenoviral vectors. Following in vivo vector delivery, persistence and function studies will be done as described above.

Aim 2. Hypothesis: systemic delivery of the δ-SG gene can be mediated by rAAV vectors through intra-artery or intra-ventricle injection.

rAAV vectors containing δ-SG gene will be delivered systemically into the hamster model. The overall vector distribution and transduction efficiency will be investigated in different muscles as well as in other tissues. More importantly, improvement of muscle histology, general health and life-span will also be evaluated.

Aim 3. Hypothesis: a dystrophin mini-gene lacking the central rod domain will improve the function of dystrophin-deficient muscle when delivered by AAV vectors.

We have recently obtained a highly truncated 3.7 kb micro-dystrophin construct (form our collaborator in Japan [97]), consisting of primarily the amino- and carboxyl-terminal regions of dystrophin with only one central

rod domain remaining (see Fig. 8 in Section C.2.2). This truncated construct was tested in an adenovirus (Ad) vector to exhibit certain functionality. No further study was carried out due to the immune complications caused by the vector system. We will study the *in vivo* functionality of the 3.7 kb micro-dystrophin gene in rAAV vectors following i.m. injection into *mdx* mice. We will examine restoration of the dystrophin associated protein complexes, improvement of muscle histology, and recovery of physiological function (Muscle Physiology Core) of *mdx* mouse muscle after high level expression of the rAAV construct.

B. Background and Significance:

B.1. AAV Vectors

AAV is a defective parvovirus that requires essential helper functions from other viruses such as adenovirus (Ad) to efficiently reproduce its progeny[8, 10]. AAV is nonpathogenic and has no etiologic association with any known diseases[9]. This safety feature should minimize the liability to the gene therapy patients. AAV is capable of infecting a broad range of mammalian cell types and tissues. Upon infection, the virus integrates into the host chromosome and persists until a suitable helper virus co-infects the cell. The only *cis*-acting sequence required for efficient integration into the host chromosome is the 145 base pair inverted terminal repeat (ITR) at each end of the AAV genome. This sequence also serves as the origin of DNA replication and packaging signal for viral DNA encapsidation[120]. Recombinant AAV (rAAV) vectors have eliminated all the viral sequences except the 145 bp inverted terminal repeats (ITRs). The removal of all viral genes adds another safety feature that not only prevents the generation of wild type helper virus via homologous recombination [99] but also mitigates the possibility of immune reactions caused by undesired viral gene expression[1, 20, 39, 71, 82, 117], a significant problem seen with other viral vector systems [123-125] (see below).

The commonly used production method for rAAV is the two-plasmid (vector/packaging) transient co-transfection system [5, 76, 99, 103, 111, 118]. The vector plasmid contains the foreign transgene(s) that is flanked by the 145 bp ITRs. The packaging plasmid contains all the AAV genes (Rep gene for viral DNA replication and Cap gene for encapsidation) but not the ITRs. The AAV gene products are supplied in trans without homologous recombination and packaging of wild type coding sequences, despite some reports documented emergence of low level wt AAV by non-homologous recombination[2]. While generally successful, the above system has at least two major drawbacks. One is the inconvenience of transient co-transfection regime. The other problem is the requirement of helper adenovirus. Even though Ad can be removed during the purification, it still poses a contamination risk. Generation of efficient packaging cells harboring both functional AAV genes and the vector DNA will offer solutions to the first problem [21, 106](also see our preliminary results), while employment of mini Ad plasmid containing only the necessary helper functions other than the entire Ad virus should prevent the second problem of Ad contamination. Recently, we have reported a high titer rAAV production method completely free of Ad virus by using a plasmid containing only the essential Ad helper genes[38, 118]. Using such three-plasmid transfection method, one can obtain Ad-free, high titer and high purity rAAV viral stocks of up to 10^{10} to 10^{11} transducing units (t.u.)/ml (up to 10^{13} to 10^{14} viral particles/ml), approaching wild type AAV yields. With the recent improvement in methodology[76, 118], rAAV vector production should no long be the bottle neck for the application of this vector system in preclinical and clinical studies[110].

AAV vectors can efficiently transduce both dividing and non-dividing cells. Transduction of proliferating cells has been well documented[10, 43, 74, 83, 115, 116]. However, despite earlier speculation and observations[42, 115], the first solid evidence of transducing non-dividing cells by rAAV vectors came from our in vivo studies in rat brains[68]. We have unequivocally demonstrated long term transduction of neuronal cells in different areas of adult rat brain using rAAV vectors, containing either a LacZ reporter gene or a therapeutic tyrosine hydroxylase gene in a Parkinsonian rat model[68]. Subsequently, we have shown that an rAAV-LacZ vector delivered through coronary artery achieved efficient gene transfer in pig heart muscle for over 6 months without significant decline of the transgene expression[69]. Recently, we and others have shown that AAV vectors, when injected in the mature muscle of mice, demonstrated sustained expression of LacZ gene up to 19 months, with molecular characterization of the vector suggesting integration as a mechanism of persistence[117]. Besides brain and muscle, other predominantly non-dividing tissues efficiently transduced by AAV vectors include liver, spinal cord and eye. Very relevant to this proposal is AAV vector's capability to bypass capillary blood vessel (pore size 20-30 nm)[94] and basal lamina of myofibers to infect the mature muscle cells. This function may partly due to the small particle size of AAV(20 nm), contrary to other viral vectors such as Ad (70-100 nm) and herpes viruses (120-300 nm), which poorly transduce mature muscle cells because of the basal lamina barrier and other factors[63].

Vector DNA copy number per genome will be determined by probing the PCR products with radio-labeled probes against the beta-actin PCR product, as well as against standard DNA copy number control of known amount plasmid DNA.

ii) Investigation of vector DNA kinetics and genomic integration by Southern blot. Although semi-quantitative PCR is a quick and convenient method to assay vector DNA in large number of samples, such as DNA from different tissues of a number of animals, this method can not distinguish double stranded vector DNA from single stranded. In addition, it can not tell if the DNA is integrated into chromosome DNA, or it is just in free episomal form. Therefore, Southern analysis is required to determine the fate of the vector DNA as a function of time post vector delivery. We will follow our previous strategy and protocols to determine the fate as well as copy number of vector DNA in both cardiac and skeletal muscle tissues (See Fig.6 & 11, and reference XX). The information can help us to obtain insight into the transduction mechanism and evolve new strategies to improve the process.

Aim 3. Hypothesis: a dystrophin mini-gene lacking the central rod domain will improve the function of dystrophin-deficient muscle when delivered by AAV vectors.

Rationale and experimental design: We will study the *in vivo* functionality of the 3.7 kb mini-dystrophin gene in rAAV vectors following injection into *mdx* mice. As discussed in Preliminary Studies, we have recently cloned a highly truncated dystrophin gene into AAV vector. This truncated dystrophin cDNA consists of primarily the amino- and carboxyl-terminal regions along with a single rod domain, and is driven by the RSV promoter (Fig. 8). This truncated construct has restored some functionality of dystrophic muscle in mdx mice when delivered by an Ad vector. The functional recovery is primarily indicated by the reconstitution of both dystroglycan and sarcoglycan complexes upon localization of the mini-dystrophin onto the muscle cell membrane. However, other beneficial effects can not be evaluated due to Ad vector's toxicity and cellular immune reactions. Since AAV vector features many advantages that Ad vectors lack, we will extend the test of the truncated dystrophin gene beyond biochemical functionalities. If encouraging results are obtained, we will further develop this line of experimentation by testing this truncated dystrophin construct with muscle-specific promoter MCK. Because mdx mice do not suffer life threatening muscle function loss (unlike Bio14.6 hamster) and they enjoy almost normal life span, we plan not to perform whole body systemic vector delivery through intraventricular injection. Instead, We will limit our experiments to local intramuscular and intra-artery injections. In addition, we will focus our effort on the leg skeletal muscles, particularly the tibialis anterior (TA) muscle for both histological and physiological function studies, in addition to biochemical characterization.

D.3.1. ***Vector delivery though intramuscular and intra-artery injection.***

First, we will examine restoration of the dystrophin associated protein complexes and recovery of physiological function of *mdx* mouse muscle after high level expression of the construct. After intramuscular and intra-artery vector injection with different doses, mice will be sacrificed at different time points (3-week, 2-month and 4-month) and muscle tissue collected as described in previous Aims.

D.3.2. ***Functional assays:***

i) Immunofluorescent staining with a battery of antibodies against various proteins on the sarcolemma of muscle cells. Again, the working protocols obtained from Dr. Eric Hoffman (Project 4) will be utilized through out the entire process. First, polyclonal antibody against the C-terminus of dystrophin[56], or Dystrophin Kit (Novocastra Laboratories) will be used to detect the localization of the highly truncated dystrophin ΔDysM3. We expect to see the appearance of this protein on the myofiber membrane, since it has been shown with an Ad vector. Second, antibodies against dystroglycan complex proteins, such as β-dystroglycan (β-DG) and α1-syntrophin (α1-SYN), will be used to detect the restoration of this complex, with which the dystrophin is associated. Third, antibodies against sarcoglycan complex proteins, such as α-sarcoglycan and δ-sarcoglycan (δ-SG), will be used to reveal the restoration of this complex. If biochemical evidence shows the structural recovery of these muscle membrane proteins, we will further examine the expected functional recovery in the muscle tissue. Methods are detailed in Aim 1.

ii) Histological staining of the vector treated muscle tissue should reveal improvement of the histopathology. We should see less centrally nucleated myofibers, more myofibers with normal and even size, and less connective tissue overgrowth. Methods are as in Aim 1.

iii) Muscle strength test should also indicate increased force in both isomeric and eccentric assays. Again, tibialis anterior muscle will be the target tissue in these assays, and statistically significant number of samples will be chosen. According to data in the literature, we will use 8 animals in each test group and the average force is analyzed with ANOVA test. Methods are detailed in Appendix A, and Aim 1.

Potential pitfalls and alternative strategies:

First, if expression of the truncated dystrophin gene in mdx mice causes CTL response and significant lymphocy infiltration and loss of vector transduced cells are observed, we will use similar strategies discussed in Aim 1 to counteract the problem. For example, alternative tissue-specific promoter and immunosuppresants will be employed. Again, we will consult Dr. Paula Clemens (Project 2) on these issues.

Second, no significant functional recovery is rendered by this highly truncated dystrophin construct. We anticipat this scenario may happen because this construct is highly abnormal, with only one rod region. However, many groups are working on other novel deletion constructs. These additional constructs could be tested in our rAAV vector system. For example, it is known that certain region of the C-terminus can be deleted without overt functional loss[17]. Removal of this distal portion of the C-terminal region can empty some space for additional rod domains to be incorporated. We have calculated that 5 to 6 rod domains can be restored after removal of the non-essential C-terminal region, and the construct can still be packaged into rAAV vector with CMV promoter and a polyA site. Notwithstanding, the most practical and also important strategy for us to shed light on this functional issue is to test the construct in neonatal mdx mice. It is possible that in adult muscle, the damage is already done and can not be effectively reversed, although we this is unlikely. In fact, previous published significant functional correction data are either done in neonates or in transgenic mice. Although we proposed all of our experiments in mature animals, this finding gives us a justification to test the construct in neonates as an alternative strategy. Dr. Johny Huard (Project 3) has tested our rAAV-LacZ vector in neonate mice and found that efficient transduction can be achieved in the muscle tissue (personal communication). We will also consult Dr. Paula Clemens on this issue as numerous neonatal Ad vector delivery experiments are planned in Project 2. With our preliminary in vivo rAAV transduction data and our expertise and combined collaborative efforts, we are poised to rigorously test the feasibility of rAAV vectors in muscle disease gene therapy.

E. Human Subjects ---Not applicable

F. Vertebrate Animals

1) Dystrophic hamsters (strain Bio14.6) and the normal control hamster (strain F1B) are purchased through Bio-Breeder, Fitchburg, MA. This is the only animal model available for δ-sarcoglycan deficiency and LGMD studies proposed in this Project.

2) Dystrophic mice (strain mdx) and the normal control mice C57BL/10 are purchased from Jackson Laboratory. They are most widely used experimental animals for DMD studies.

3) The institutional guidelines for the use and care of animals in research are assured by Animal Welfare Assurance No. A3187-01 at the University of Pittsburgh and the proposed studies (protocol # 0398039) have been approved under those guidelines. The animal care facility for both hamsters and mice are supervised by qualified veterinarians, and the animals are maintained by a group of trained animal care technicians. Animals will be euthanized by cervical dislocation after anesthesia with Avertin 2.5% 0.4 mg/g I.P, or by carbon dioxide asphyxiation, consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Association.

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ducing neurogenesis from resident neural progenitor cells in the adult forebrain. The induction of striatal neurogenesis in particular may have significant implications in diseases of striatal neuronal loss, such as Huntington's disease and the striatonigral degenerations.

Supported by the Mathers Charitable Foundation, the National Multiple Sclerosis Society, and NIH grants R01 NS29813, R01 NS33106, and P50 HL59312.

53. Efficient Functional Correction of Muscular Dystrophy in *mdx* Mice by AAV Vectors Carrying Novel Human Mini-dystrophin Genes

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Duchenne muscular dystrophy (DMD), caused by recessive mutations in dystrophin gene, affects one of every 3500 born males. No treatment is available for DMD, which is the most common and lethal progressive muscle disorder characterized. Genetic therapeutic approaches using primarily myoblast transplantation or adenovirus-mediated gene transfer, have met with limited success. On the other hand, AAV as the only non-pathogenic viral vector currently available, has been successful in establishing efficient and long-term gene expression without significant immune response or toxicity in vivo. Contrary to other viral vectors, AAV readily bypasses the extracellular barrier in muscle due to its small particle size (20 nm) and transduces myofibers of various maturity. Currently, AAV vectors have offered the best gene transfer efficiency and longevity among all viral and non-viral vectors tested in muscle tissues. The unparalleled efficiency and safety of the vector system in muscle have led to its utility in gene therapy at a increasing pace, for genetic muscle disorders as well as for metabolic diseases involving genes of smaller size (< 4.5 kb). However, the small package size (5 kb) has precluded its utility for dystrophin gene (14 kb cDNA).

In this study, we show for the first time the successful gene therapy of DMD using AAV vectors. We have created a series of novel mini-dystrophin genes (3.8 kb to 4.2 kb) that readily package into AAV along with an MCK (muscle-specific creatine kinase) promoter to assure tissue-specific transgene expression. When injected into the hindleg muscle (gastrocnemius) of mdx mice (a DMD animal model), two of the mini genes resulted in efficient and stable expression (3 months, current duration of the study) in a widespread (50% to 88%) of the myofibers across the transverse sections of the vector treated muscle. Immunofluorescent staining of the consecutive sections, using antibody against a, b, and g sarcoglycans, revealed the restoring the missing dystrophin-associated protein complexes onto the plasma membrane. Furthermore, AAV-transduced mdx muscle exhibited normal polygonal morphology of the myofibers with consistent sizes. More importantly, Counterstaining of nuclei with DAPI dye showed more than 98% of the myofiber nuclei located at the normal peripheral positions, indicating the absence of muscle degeneration and regeneration. H & E staining revealed normal histology and lack of fibrosis and infiltration in the vector transduced area. Finally, myofiber membrane leakage test using a vital dye Evans blue, showed indistinguishable membrane integrity of AAV treated myofibers from those of healthy mice. Thus, we have demonstrated for the first time that the dystrophin gene can be shortened to approximately 1/3 of its 11 kb coding sequence without loosing the functions of preventing muscle tissues from dystrophic phenotypes. These findings further define the minimal functional domains of dystrophin, and more importantly render a promising new avenue for DMD gene therapy using AAV vectors.

54. Engineered Neural Progenitor Cells for Gene Therapy of *twitcher*, the Murine Model of Globoid Cell Leukodystrophy

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Globoid cell leukodystrophy (GLD or Krabbe disease) is a degenerative disorder of the nervous system affecting primarily infants and caused by mutations of the gene encoding the lysosomal enzyme galactocerebrosidase (GALC). Bone marrow transplantation may counteract the disease progression in late-onset forms but no effective treatment is presently available for early-onset GLD. Twitcher is the murine model of GLD and we are developing experiments to test whether brain transplantation of neural progenitor cells overexpressing GALC may affect the twitcher phenotype. Initially we prepared an MMLV-based ecotropic packaging cell line transducing the GALC gene. This retrovirus was used for transduction of rat neural progenitor cells immortalized by a temperature-sensitive variant of the SV40 large T antigen (ST14A cells). Transduction of ST14A cells led to a 4-fold increase of GALC activity after antibiotic selection (from 15.6 to 63.3 nmol/mg/hr; Torchiana et al, 1998). Subsequent experiments based on injection of ST14A/GALC cells in the vicinity of the lateral ventricle in 12-14 day old twitcher mice (symptoms start around day 20 and animals usually die two weeks later) did not modify disease progression. Injection of syngeneic fibroblasts overexpressing GALC, however, was accompanied by a slight prolongation of survival, suggesting that immunological responses could decrease survival of allogeneic ST14A cells after transplantation in mouse brain. To overcome this, we have now isolated and grown into culture neural progenitor cells from a newborn heterozygous twitcher mouse. We have also cloned GALC cDNA into lentiviral plasmid pRRLsin.PPT.hCMV.Wpre and assembled by cotransfection of plasmids pCMVdR8.74 and pVSV-G into 293 T cells a lentiviral vector for GALC transduction. The viral supernatant (80 ng /ml of p24, as determined by immunocapture) was used to infect HeLa cells, causing an 80 fold increase (from 0.07 to 8.9 nmol/mg/hr) of GALC activity in the absence of antibiotic selection. Transduction of syngeneic neural stem cells with this lentiviral vector should offer the appropriate conditions to evaluate if this approach is therapeutically relevant. Alternatively, direct injection of the viral supernatant in the CNS or transduction of hematopoietic stem cells will be considered for gene therapy of twitcher.

55. *In Vivo* Migration of Transplanted Myoblast Requires Matrix Metalloproteinases Activity: Improving Muscle-targeted Gene Therapy

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Myoblast transplantation is a potential treatment of the Duchenne Muscular Dystrophy, and of many other genetic diseases and acquired diseases. The success of this method, as a therapeutic intervention, however, depends on several factors. Not the least being the movement of transplanted cells throughout the host muscle in order to disseminate the introduced normal gene product, in particularly when this product has a structural function as does dystrophin. Using a new technique to assess, in vivo, myoblast migration, we have confirmed previous results showing that the C2C12 myoblast cell line exhibits a higher migratory capacity, than do the primary myoblasts. To test the hypothesis

Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in *mdx* mouse model

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Edited by William S. Sly, St. Louis University School of Medicine, St. Louis, MO, and approved September 20, 2000 (received for review July 19, 2000)

Duchenne muscular dystrophy (DMD) is the most common and lethal genetic muscle disorder, caused by recessive mutations in the dystrophin gene. One of every 3,500 males suffers from DMD, yet no treatment is currently available. Genetic therapeutic approaches, using primarily myoblast transplantation and adenovirus-mediated gene transfer, have met with limited success. Adeno-associated virus (AAV) vectors, although proven superior for muscle gene transfer, are too small (5 kb) to package the 14-kb dystrophin cDNA. Here we have created a series of minidystrophin genes (<4.2 kb) under the control of a muscle-specific promoter that readily package into AAV vectors. When injected into the muscle of *mdx* mice (a DMD model), two of the minigenes resulted in efficient and stable expression in a majority of the myofibers, restoring the missing dystrophin and dystrophin-associated protein complexes onto the plasma membrane. More importantly, this AAV treatment ameliorated dystrophic pathology in *mdx* muscle and led to normal myofiber morphology, histology, and cell membrane integrity. Thus, we have defined minimal functional dystrophin units and demonstrated the effectiveness of using AAV to deliver the minigenes *in vivo*, offering a promising avenue for DMD gene therapy.

Duchenne muscular dystrophy (DMD) is an X-linked genetic muscle disease affecting 1 of every 3,500 males born (1–3). The progressive muscle degeneration and weakness usually confine the patients to wheelchairs by their early teens and lead to death by their early twenties. DMD is caused by recessive mutations in the dystrophin gene, the largest gene known to date, which spans nearly 3 million bp on the X-chromosome (2) with a high rate of *de novo* mutations. Dystrophin is an enormous rod-like protein (427 kDa) localized beneath the inner surface of myofiber plasma membrane in both skeletal and cardiac muscles (3). Dystrophin functions through four major structural domains. The N-terminal domain binds to the F-actin of cytoskeletal structures, whereas the C-terminal cysteine-rich (CR) domain along with the distal C terminus (CT), anchors to the plasma membrane via dystrophin-associated protein (DAP) complexes. The central rod domain contains 24 triple-helix rod repeats and four hinges (4). Thus, dystrophin crosslinks and stabilizes the muscle cell membrane and cytoskeleton. The absence of a functional dystrophin results in the loss of DAP complexes and causes instability of myofiber plasma membrane. These deficiencies in turn lead to chronic muscle damage and degenerative pathology.

Because of the lack of effective treatment for DMD, novel genetic approaches including cell therapy and gene therapy have been actively explored. However, clinical trials of myoblast transplantation have met with little success, owing to the poor survival of the transplanted cells (5). Gene therapy as an alternative strategy has been extensively studied in animal models. Somatic gene transfer using both nonviral DNA vectors carrying dystrophin cDNA (6) and RNA/DNA oligonucleotides (7) achieved transgene expression but with very limited efficiency. Adenovirus-based vectors have been successfully tested in dystrophic animal models (8, 9). Nonetheless, the immunogenicity and inefficiency of infecting mature muscle cells remain major hurdles to overcome before the vector can be safely used in humans.

Adeno-associated virus (AAV) vectors are the only viral vector system that is based on a nonpathogenic and replication defective virus (10). AAV vectors have been successfully used to establish efficient and long-term gene expression *in vivo* in a variety of tissues without significant immune response or toxicity (11–14). Unlike other viral and nonviral vectors, AAV readily bypasses extracellular barriers because of its small viral particle size (20 nm) that facilitates efficient transduction of muscle myofibers of various maturity (15). Currently, AAV vectors offer the best gene transfer efficiency and longevity among all viral and nonviral vectors tested in muscle tissues. The unparalleled efficiency and safety have led to an increasing interest in AAV-mediated gene therapy for genetic muscle disorders (16–18) as well as for metabolic diseases. However, until recently (19, 20) a major obstacle for AAV vectors is the limited packaging size that only allows for genes smaller than 4.5 kb (13, 17, 21, 22), therefore precluding such a large gene as dystrophin with a cDNA of 14 kb. Here we have created miniature versions of dystrophin genes ideal for AAV vector-mediated DMD gene therapy.

Materials and Methods

Construction of Minidystrophin Genes and AAV Vector Production. Minidystrophin constructs were made mainly by the PCR cloning method using Pfu polymerase (Stratagene) and human dystrophin cDNA (GenBank NM 004006) as the template. For consistency, the numbering of the nucleotide only includes the 11,058 bp dystrophin protein coding sequence. As depicted in Fig. 1, minigene Δ3849 contains nucleotides 1–1668 (N terminus, hinge 1, and rods 1 and 2), 8059–10227 (rods 22, 23, and 24, hinge 4, and CR domain), and 11047–11058 (the last 3 aa of dystrophin). Similarly, minigene Δ3990 contains nucleotides 1–1668, 7270–7410 (hinge 3), 8059–10227, and 11047–11058. Finally, minigene Δ4173 contains nucleotides 1–1992 (N terminus, hinge 1, and rods 1, 2, and 3), 8059–10227, and 11047–11058. The above constructs were made by blunt-end ligation of the Pfu-amplified PCR products of each individual segment, so that all

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Abbreviations: AAV, adeno-associated virus; DMD, Duchenne muscular dystrophy; CR, cysteine-rich; CT, C terminus; DAP, dystrophin-associated protein; MCK, muscle-specific creatine kinase; CMV, cytomegalovirus; DAPI, 4′,6-diamidino-2-phenylindole; IF, immunofluorescent.

See commentary on page 13464.

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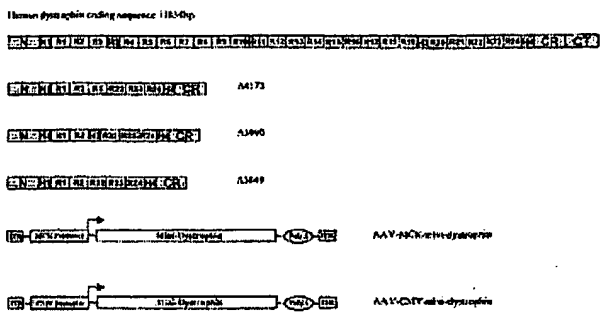

Fig. 1. Construction of highly truncated minidystrophin genes. Dystrophin has four major domains: the N-terminal domain (N), the CR domain, the CT domain, and the central rod domain, which contains 24 rod repeats (R) and four hinges (H). The minidystrophin genes were constructed by deleting a large portion of the central rods and hinges and nearly the entire CT domain (except the last 3 aa). The minidystrophin genes subsequently were cloned between an MCK promoter (or a CMV promoter) and a poly(A) sequence in AAV vectors.

of the protein coding sequences are precisely spliced together in-frame. The minidystrophin genes then were subcloned into an AAV vector plasmid containing a muscle-specific creatine kinase (MCK) promoter, a 595-bp *Hin*dIII/*Bst*EII fragment from plasmid p(+enh206)358MCKCAT (23), and a 60-bp small poly(A) signal sequence (24), resulting in vector constructs AAV-MCK-Δ3849, AAV-MCK-Δ3990, and AAV-MCK-Δ4173. Similarly, the minigenes also were cloned into an AAV vector plasmid containing a cytomegalovirus (CMV) promoter (620 bp) and the small poly(A) signal sequence to generate vector constructs AAV-CMV-Δ3849 and AAV-CMV-Δ3990.

The recombinant viral vector stocks were produced precisely according to the three-plasmid cotransfection method as described by Xiao *et al.* (25). The AAV viral vectors were subsequently purified twice through CsCl density gradient ultracentrifugation using the previously published protocols (26). The vector titers of viral particle number were determined by DNA dot blot method (26) and were approximately 5×10^{12} to 1×10^{13} viral particles per ml.

Mice and Vector Administration. All experiments involving animals were approved by the University of Pittsburgh Animal Care and Use Committee. The healthy mice *C57/B10* and dystrophic mice *mdx* were purchased from The Jackson Laboratory. The 10-day-old *mdx* pups or 50-day-old *mdx* adult mice were injected into the hindleg gastrocnemius muscle with 50 μl (5×10^{10} viral particles) of different AAV minidystrophin vectors. Muscle samples were collected for examination at various time points after vector injection.

Immunofluorescent (IF) Staining. Muscle cryosections of 8 μm thickness were immunofluorescently stained with the Mouse-on-Mouse Kit from the Vector Laboratories according to the manufacturer's protocol, except that the cryosections were immediately treated with the blocking buffer without the fixation step (27). Monoclonal antibodies against dystrophin (NCL-Dys3 and NCL-Dys2) and against α-, β-, and γ-sarcoglycans (NCL-a-SARC, NCL-b-SARC, and NCL-g-SARC) were purchased from NovoCastra Laboratories (Burlingame, CA). Muscle cell nuclei were counterstained with 0.01% 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min. Photographs were taken with a Nikon TE-300 fluorescent microscope.

***In Vivo* Myofiber Plasma Membrane Integrity Test.** Evans Blue dye (10 mg/ml PBS) was injected into the tail vein of C57/B10 mice, *mdx* mice, and AAV vector-treated *mdx* mice at the dose of 0.1 mg/g of body weight (28). After dye injection, mice were allowed continuous swimming for 20 min. At 15 h after Evans Blue injection, muscles were collected and cryosectioned. Evans Blue dye-positive myofibers were observed under the fluorescent microscope with rhodamine filters.

Results

Construction of Minidystrophin Genes. To explore the feasibility of using AAV vectors for DMD gene therapy, we have devised strategies to create minidystrophin genes, which are small enough to be packaged into AAV vectors, and yet retain the essential functions needed for protecting muscle from the pathological symptoms. Previous studies on patients with mild muscular dystrophy revealed that although they endured large in-frame deletions in the central rod domain of dystrophin, those patients suffered only slight symptoms (29–31). This phenomenon suggests that a major portion of the rod domain is dispensable. In addition, transgenic studies in *mdx* mice showed that two deletion mutants in the CT, one lacking exons 71–74 and the other lacking exons 75–78, displayed full functions in preventing dystrophic phenotypes (32). This result suggests that the CT domain also may be dispensable. In contrast, N-terminal deletions variably impair dystrophin functions (33). Based on the above observations, we have created by rational design several minigenes, in each deleting up to 75% of the central rod domain (19 of the 24 rods; 2 of the 4 hinges) as well as nearly the CT domain (exons 71–78) (Fig. 1).

These minigenes have enabled us to re-examine a previous hypothesis that a dystrophin could not be made smaller than one-half of its full length without causing muscular dystrophies (34). Our minidystrophin genes, as small as only one-third of the 11-kb full-length dystrophin coding sequence, are significantly smaller than the 6.3-kb Becker-form minidystrophin gene (29) that was widely used in transgenic and gene therapy studies in *mdx* mice (8). To ensure sufficient physical flexibility of the minidystrophin protein, all of our constructs such as Δ3849 still retain at least five rod repeats (R1, R2, R22, R23, and R24) and two hinges (H1 and H4) in the central rod domain (Fig. 1). Construct Δ3990 has an additional hinge (H3), whereas construct Δ4173 contains an additional rod (R3) (Fig. 1). The rationale of deleting central portion of the rod domain while preserving both distal rod repeats in our minigenes is based on the fact that those distal repeats were naturally retained in the mild Becker muscular dystrophy patients, who had large in-frame deletions in the rod domain (29–31). The above minidystrophin genes were packaged into AAV vectors under the control of a strong promoter CMV (CMV immediate early promoter) (Fig. 1). To ensure muscle-specific expression, the minigenes also were packaged into AAV vectors under the control of an MCK promoter (23) (Fig. 1).

Restoration of DAP Complexes. Because our minidystrophins lack nearly the entire distal CT domain, this prompted us to investigate whether those constructs still retain the major biochemical functionality including submembrane localization and interaction with DAP complexes. We initially injected the AAV MCK minidystrophin vectors into the hindleg muscle (gastrocnemius) of 10-day-old *mdx* mice. At 3 months and 6 months after vector injection, the muscles were collected for evaluation of minidystrophin expression and biochemical restoration of the DAP complexes, which were absent because of the primary deficiency of dystrophin. IF staining on thin sections of AAV-treated muscles, using an antibody (Dys3) specific to human dystrophin, revealed widespread vector transduction and correct submembrane location of the minidystrophins in a majority of the myofibers, especially in muscles treated with AAV vectors containing minigene Δ3849 or Δ3990 (Figs. 2 *a* and *b* and 3*a*). As

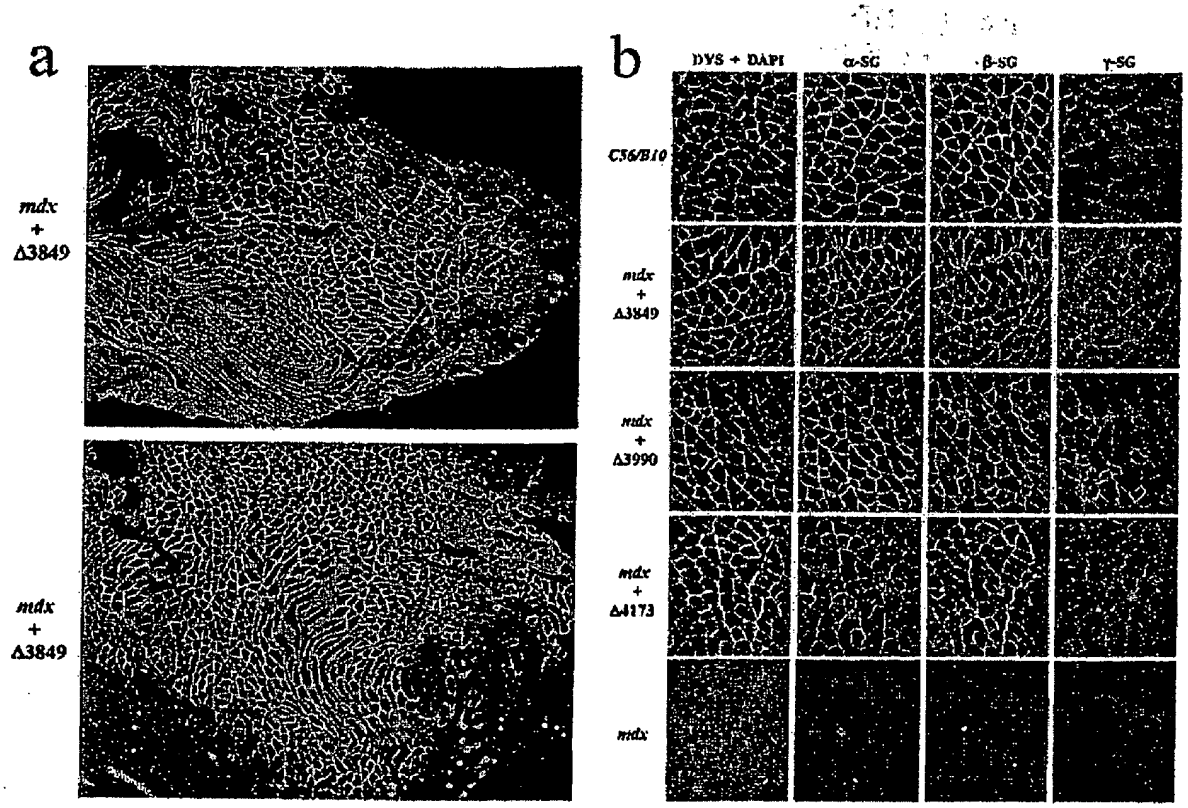

Fig. 2. IF analysis of the dystrophin and DAP complexes in gastrocnemius muscle. (*a*) Cryosections of *mdx* muscle, at 3 months after treatment with construct AAV-MCK-Δ3849 or AAV-MCK-Δ3990, were IF-stained with an antibody against dystrophin (green) and then counterstained for cell nuclei with DAPI (blue). Photos were taken with a ×4 microscope lens. Note the widespread minidystrophin expression and peripheral nucleation in a majority of the myofibers. Also note the extensive central nucleation in minidystrophin-negative areas. (*b*) Cryosections of muscles from 15-week-old normal C57/B10 mice, from *mdx* mice treated either with vector AAV-MCK-Δ3849, AAV-MCK-Δ3990, or AAV-MCK-Δ4173, or from untreated *mdx* mice were IF-stained with antibodies for dystrophin (green) and counterstained with DAPI (blue) for nuclei (DYS + DAPI). Note the lack of central myonuclei. The consecutive sections also were stained with antibodies for α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), and γ-sarcoglycan (γ-SG). Photographs were taken with a ×20 lens.

expected, the equivalent muscle from the age-matched healthy C57/B10 mice showed an indistinguishable dystrophin staining pattern, when stained with an antibody (Dys2) that recognizes both mouse and human dystrophin CT region (Fig. 2*b*). As expected, this antibody (Dys2) failed to stain the AAV-treated *mdx* muscle because of deletion of the CT region in our minidystrophin genes (data not shown). This result further confirmed the identity of minidystrophins that were derived from the AAV vectors. Consistently, the untreated *mdx* control muscle showed no dystrophin staining (Fig. 2*b*) except the very few somatic revertant myofibers recognized by Dys2 antibody. Furthermore, injection of AAV minidystrophin vectors into the adult *mdx* muscle (gastrocnemius) showed similar results when examined for dystrophin expression at 2 and 4 months after injection of AAV MCK vectors (Fig. 3 *c–f*) or at 6 months after injection of AAV CMV vectors (Fig. 3 *g* and *h*). Importantly, there was no cytotoxic T lymphocyte destruction against the myofibers that persistently expressed minidystrophins of human origin from AAV vectors, either driven by a CMV promoter or by a muscle-specific MCK promoter.

We next examined whether the minidystrophins were functional in restoring the missing DAP complexes on the myofiber plasma membrane, including the sarcoglycan complex, which is not found in untreated dystrophic muscle because of the primary deficiency of dystrophin. IF staining using three antibodies against α, β, and γ sarcoglycans, respectively, showed positive results in all of the consecutive thin sections adjacent to those stained with dystrophin antibodies (Fig. 2*b*). These results provided evidence of biochemical functionality of the minidystrophins, which lack the CT domain but are still capable of interacting with the DAP complexes.

Amelioration of Dystrophic Pathology. To further investigate the functionality of our minidystrophins, it is essential to demonstrate that they can protect muscle from the pathological phenotypes. The onset of the pathology in *mdx* mice starts at around 3 weeks of age with massive waves of myofiber degeneration/regeneration. This process is characterized by the presence of central nuclei in myofibers, a primary pathological sign of muscular dystrophies. The absence or reduction of central nucleation after gene therapy would suggest that the therapy is successful. Therefore, we initially chose to test the AAV minidystrophin constructs in young *mdx* mice (10 days old) before the onset of central nucleation, to see whether muscle degeneration/regeneration can be prevented.

Histological examination of the *mdx* muscles at 3 and 6 months after AAV minidystrophin treatment, which was before the onset of central nucleation, showed nearly exclusive (≈98%) peripheral nucleation in the minidystrophin-positive myofibers, as revealed by dystrophin immunostaining and myonuclei coun-

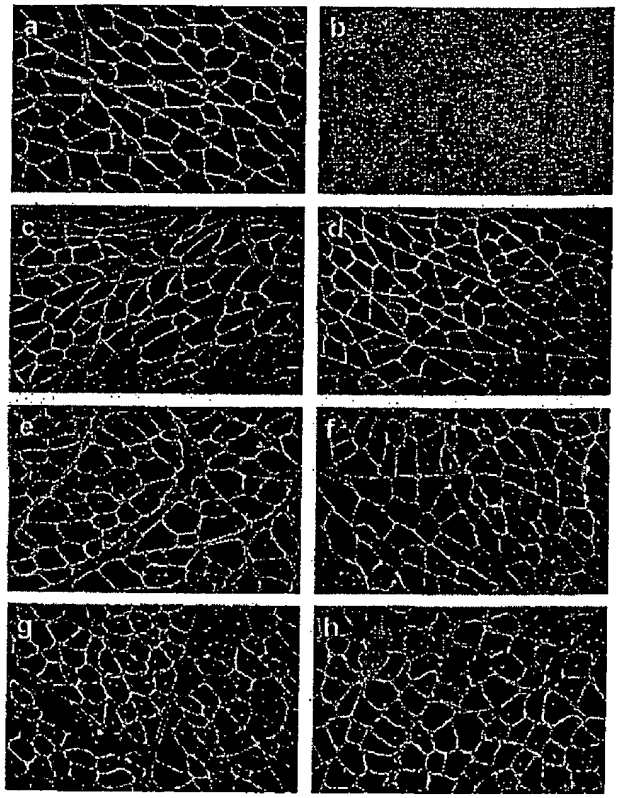

Fig. 3. Long-term minidystrophin expression in *mdx* mice treated at a young age (*a*) or as adults (*c–h*) with AAV vectors containing different minigenes under the control of different promoters. IF staining of minidystrophin (green) and myonuclei counterstaining with DAPI (blue) were performed on gastrocnemius muscles isolated from (*a*) MCK-Δ3849-treated 10-day-old *mdx* for 6 months, (*b*) untreated 6-month-old *mdx*, (*c*) MCK-Δ3849-treated adult *mdx* for 2 months, (*d*) MCK-Δ3990-treated adult *mdx* for 2 months, (*e*) MCK-Δ3849-treated adult *mdx* for 4 months, (*f*) MCK-Δ3990-treated adult *mdx* for 4 months, (*g*) CMV-Δ3849-treated adult *mdx* for 6 months, and (*h*) CMV-Δ3990-treated adult *mdx* for 6 months.

terstaining with DAPI [Figs. 2 *a* and *b* (first column) and 3*a*; Table 1]. The mutual exclusivity between minidystrophin expression and central nucleation in the vector-treated *mdx* muscle precisely mirrored that of the normal muscle (Fig. 2*b* and Table 1). In addition, the myofibers positive for minidystrophin expression also exhibited consistent myofiber sizes and polygonal shapes indistinguishable from those of the normal muscle (Fig. 2). By contrast, the untreated *mdx* muscle showed extensive (75.4%) central nucleation (Table 1), with additional signs of dystrophic pathology, including wide variation of myofiber sizes, round myofiber shapes, and fibrosis (Fig. 2*b*). Hence, AAV vector treatment prevented dystrophic pathology and led to normal histology in terms of peripheral nucleation, consistent myofiber size, and lack of fibrosis in the minidystrophin-positive areas. These results unequivocally demonstrated the absence of muscle degeneration because of the therapeutic effects of our minidystrophins in young *mdx* mice.

Subsequently we also tested AAV minidystrophin vectors in treating adult *mdx* mice (45 days of age) after the onset of massive waves of degeneration/regeneration, to see whether the pathological process can be stopped or reversed. At the time of vector injection, a majority (≈75%) of the myofibers already underwent degeneration/regeneration process and displayed central nucleation. At 2 months, 4 months, and 6 months after AAV minidystrophin injection, widespread dystrophin expression was observed accompanied by normal myofiber morphology and lack of fibrosis in the dystrophin-positive areas (Fig. 3). By contrast, muscle of untreated *mdx* mice (Fig. 3*b*), or areas of treated muscle without successful vector gene transfer, manifested progressive degeneration and fibrosis. In addition, a reduction of central nucleation in minidystrophin-positive myofibers was observed (from approximately 75% before vector treatment to 35–50% after vector treatment; see Table 1). The partial reversal of central nucleation also was observed in healthy mouse muscle, where a majority of the myonuclei remained centrally located once experiencing a transient pathology such as myotoxin treatment (35). Persistence of central nucleation also was observed after treatment of adult *mdx* muscle with a gutless adenovirus vector containing the full-length dystrophin cDNA (P. Clemens, personal communication). Based on the above observations, our minidystrophin genes demonstrated therapeutic effects in ameliorating dystrophic pathology in both young and adult *mdx* muscles.

Protection of Myofiber Membrane Integrity. Plasma membrane damage and leakage in dystrophic muscle is a major physiological defect and also a major pathological cause. To determine whether AAV minidystrophin treatment would be effective in protecting plasma membrane from mechanical damage, a myofiber membrane integrity test was performed by i.v. injection of Evans Blue dye. Evans Blue is a widely used vital red fluorescent dye that is excluded by the healthy myofibers, but is taken up by the dystrophic myofibers containing leaky cell membrane caused by contractile damages. A previous study of *mdx* mice revealed that the apoptotic myonuclei were found mostly in Evans Blue dye-positive myofibers, thus correlating plasma membrane leakage and muscle cell apoptosis (28).

Initially, Evans Blue was administered into the tail vein of *mdx* mice that were treated at a young age (10 days old) with AAV vectors 3 months before. The age-matched untreated *mdx* mice and healthy C57/B10 mice were used as controls. To induce mechanical stress, the mice were allowed to exercise by continuous swimming for 20 min. Muscles then were collected and examined for dystrophin expression as well as for Evans Blue dye uptake. As expected, muscle from healthy mice revealed uniform dystrophin staining across the muscle sections and no uptake of the dye by the myofibers (Fig. 4*a*, top row). The AAV vector-treated *mdx* muscle showed results consistent with the healthy muscle, thus mutual exclusivity of dystrophin expression and dye uptake (Fig. 4*a*, three middle rows). Dye uptake (red fluorescence) was found only in myofibers that lacked minidystrophin expression in the areas not transduced by AAV vectors (Fig. 4*a*, three middle rows). By contrast, the untreated *mdx* muscle revealed absence of dystrophin and extensive dye uptake (Fig. 4*a*, bottom row). More importantly, AAV minidystrophin treatment of adult *mdx* muscle also achieved similar results in protecting myofibers from plasma membrane leakage when analyzed at 2 months and 6 months after vector injection (Fig. 4*b*). These results unequivocally demonstrated the physiological functionality of our minidystrophins in maintaining membrane integrity and protecting myofibers from mechanical damages in both young and adult *mdx* mice.

Discussion

In summary, we have presented evidence that the dystrophin gene can be successfully reduced to one-third of its 11-kb full-length coding sequence, without compromising essential functions in protecting muscles from dystrophic phenotypes. Moreover, we have demonstrated that intramuscular injection of AAV vectors carrying human minigenes can achieve efficient

Table 1. AAV minidystrophin gene transfer in young and adult *mdx* mice

Animals* and vectors	*n*	Age at vector injection	Months post injection	% Dystrophin-positive fibers	% Central nuclei†
mdx + Δ3849	4	10 days	3	56 ~ 88	1.02 (72/7,098)
mdx + Δ3990	4	10 days	3	50 ~ 80	0.99 (56/5,652)
mdx + Δ4173	4	10 days	3	15 ~ 25	0.93 (26/2,791)
mdx + Δ3849	4	10 days	6	40 ~ 60	2.80 (51/1,824)
mdx + Δ3990	2	10 days	6	35 ~ 45	2.30 (34/1,478)
mdx + Δ3849	2	50 days	2	35 ~ 50	34.76 (510/1,467)
mdx + Δ3990	2	50 days	2	35 ~ 40	34.18 (685/2,004)
mdx + Δ3849	4	50 days	4	20 ~ 25	44.24 (615/1,390)
mdx + Δ3990	4	50 days	4	20 ~ 30	46.18 (695/1,505)
C57/B10	4	No injection	N/A	100	1.45 (56/3,860)
mdx	4	No injection	N/A	<1	75.4 (238/3,160)

N/A, Not applicable.

*Untreated control *mdx* and C57/B10 mice were about 3 months old at the endpoints of experiments. AAV vectors were driven by MCK promoter.

†All numbers were collected from dystrophin-positive myofibers that were photographed after IF staining and DAPI counterstaining, except in untreated *mdx* mice, which had extensive central nucleation and very few dystrophin-positive revertant myofibers.

and long-term therapeutic effects in a major muscle group of a DMD mouse model. The long-term correction of both biochemical and physiological defects in the dystrophic muscles was realized by the persistent minidystrophin expression from AAV vectors and the apparent lack of cytotoxic T lymphocyte immune response against myofibers expressing human dystrophin.

Previous attempts to generate minigenes that were shorter than one-half of the full-length dystrophin failed to preserve the essential protective functions. The minidystrophin genes tested in adenovirus vectors by Yuasa and colleagues (36, 37), although containing both intact N- and C-terminal domains and 1–3 central rod repeats, were functionally similar to a CT dystrophin construct (Dp71) (38), thus sufficient to restore DAP complexes but insufficient to restore myofiber morphology and prevent dystrophic pathology (unpublished results). By contrast, the minidystrophin genes reported here accommodated at least five rod repeats (R1, R2, R22, R23, and R24) and two hinges (H1 and H4). To retain as many repeat units in the rod domain without exceeding the packaging limit of AAV vectors, we have enabled the deletion of nearly the entire CT (819 bp) without sacrificing the primary functions of dystrophin, for example, submembrane anchoring and interaction with DAP complexes. Our results indicate that five rods and two hinges seem sufficient to provide both length and flexibility for the central domain. This conclusion is supported by the observation that minigenes Δ3849 and Δ3990 were equally functional in preventing the dystrophic

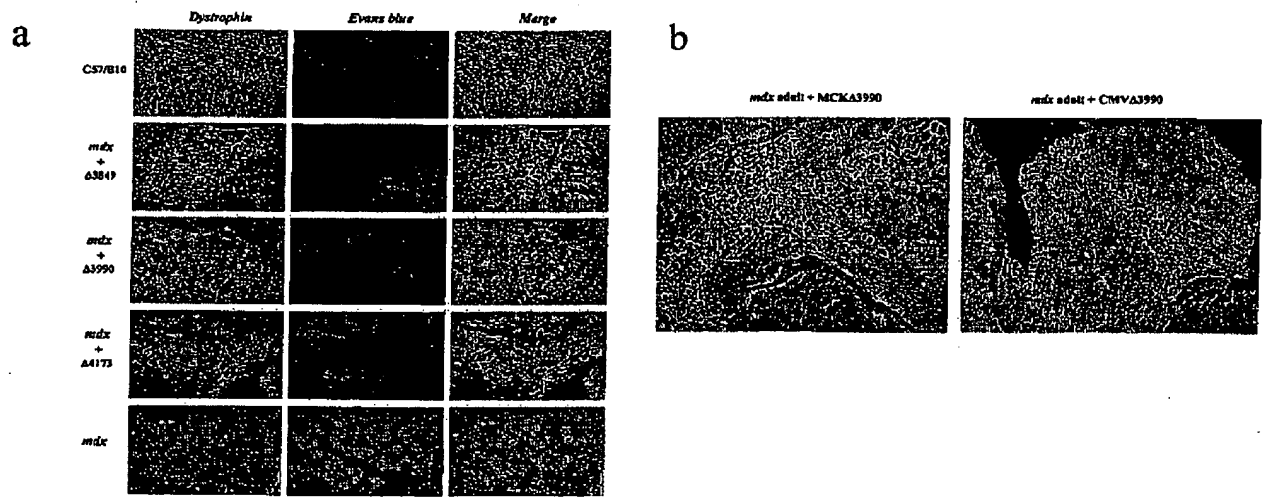

Fig. 4. Protection of muscle plasma membrane integrity by minidystrophin genes in *mdx* mice treated either at 10 days of age (*a*) or as adults (*b*). (*a*) Three months after AAV vector injection, either the treated *mdx* mice or the age-matched controls (normal C57/B10 and untreated mdx mice) were i.v. injected with Evans Blue dye. The gastrocnemius muscles then were collected and cryosectioned either from normal C57/B10 mice; from *mdx* mice treated at 10 days of age with AAV-MCK vectors Δ3849, Δ3990, or Δ4173; or from the untreated *mdx* mice. Normal dystrophin and minidystrophin expression was visualized by IF staining (*Left*, green). The leaky myofibers were visualized by the uptake of Evans Blue dye (*Center*, red fluorescence). Note the mutual exclusivity between dystrophin expression and Evans Blue dye uptake as shown by the merged images (*Right*). Photographs were taken with a ×10 lens. (*b*) Adult *mdx* gastrocnemius muscles were treated with AAV vectors containing Δ3990 minigene. Evans Blue dye tests were performed at 2 months after AAV-MCK-Δ3990 treatment (*Left*) or at 6 months after AAV-CMV-Δ3990 treatment (*Right*). Note the widespread minidystrophin expression (green) and the leaky myofibers (red), which were negative for minidystrophin staining.

phenotypes, although Δ3990 had an extra hinge (H3). Similarly, minigene Δ4173 had an extra rod (R3) but did not function better than minigenes Δ3849 or Δ3990 (Table 1). In fact, because the entire AAV-MCK-4173 vector cassette was nearly 5.2 kb in length, larger than the 5-kb packaging limit, its viral particle infectivity was impaired, which led to lower gene transfer efficiency (Fig. 4*a* and Table 1). Because a major role of dystrophin is to crosslink the myofiber cytoskeleton structure and plasma membrane, thus, to stabilize the myofiber during muscle contraction, we hypothesize that the length of the central rod domain is the critical factor in constructing functional minidystrophin genes. It is conceivable that if the minidystrophin is too short to span the sliding distance between the cytoskeleton and plasma membrane during muscle contraction, the crosslinks will be disrupted and the muscle membrane will become unstable and prone to mechanical damages.

Despite the fact that our minidystrophin genes demonstrated functionality in protecting myofiber membrane integrity even after exercises, it remains to be seen whether the protective effects withstand extreme conditions such as eccentric contractions. Further investigation also will be instructive to see whether these minigenes can restore the muscle contractile force deficit, although in *mdx* mice such deficit is not apparent unless the force output is normalized by muscle cross-sectional area. The minigenes created here are shorter than the 6.3-kb truncated dystrophin gene, which was isolated from a 61-year-old ambulant Becker muscular dystrophy patient who had very mild symptoms (29). It is plausible not to expect that the minigenes are as functional as the 6.3-kb Becker-form dystrophin gene. However, further *in vivo* functional comparisons between these genes as well as to the wild-type dystrophin gene by transgenic mouse studies will provide insightful information not only for muscle biology but also for DMD gene therapy.

Of particular importance in this study is the use of the AAV vector, which appears to be the best vector system currently available for muscle-directed gene therapy. Compared with other viral and nonviral vectors previously explored for DMD, AAV has the combined advantages of high-efficiency gene transfer, persistent transgene expression and low immunogenicity (12, 13, 17, 18, 21). The success of truncating large dystrophin gene into functional miniature versions enabled us to explore the utility of AAV vector in treating the most common and devastating genetic muscle disorder. Recent progress in stem-cell transplantation has offered a new hope for cell therapy of DMD (39). The functional dystrophin genes reported here also should find their utilities in stem-cell therapeutics after *ex vivo* gene transfer. Nevertheless, the primary advantage of AAV vectors is the direct *in vivo* gene delivery such as intramuscular injections. New developments in systemic vector delivery through the blood circulation (16) and tissue targeting of AAV vectors should render more widespread gene transfer in large groups of muscles. Finally, using AAV vectors rather than the traditional transgenic mouse technology, we have provided a more convenient and less time-consuming method to further discern the dystrophin functional domains *in vivo* and to optimize the minidystrophin genes for future clinical applications in DMD gene therapy.

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